The information previously contained on these webpages were authorized under the 2009 H1N1 Influenza Emergency Use Authorizations (EUAs). As of June 23, 2010, the EUAs have been terminated and this information is no longer current.

ViraCor 2009 H1N1 Influenza A Real-time RT-PCR Package Insert

1. Intended Use:

The ViraCor 2009 H1N1 Influenza A Real-time RT-PCR test is intended for use in ViraCor Laboratories for the in vitro qualitative detection of 2009 H1N1 influenza viral RNA in upper respiratory tract specimens (such as nasopharyngeal swabs (NPS), nasal swabs (NS), throat swabs (TS), nasal aspirates (NA), nasal washes (NW), and dual nasopharyngeal/throat swabs (NPS/TS)) and lower respiratory tract specimens (such as broncheoalveolar lavage (BAL), bronchial aspirate (BA), bronchial wash (BW), endotracheal aspirate (EA), endotracheal wash (EW), tracheal aspirate (TA), and lung tissue) from human patients with signs and symptoms of respiratory infection in conjunction with clinical and epidemiological risk factors. The testing format includes nucleic acid extraction using the bioMerieux NucliSENS® easyMag® extraction platform followed by real-time reverse-transcription PCR utilizing the Applied Biosystems 7500 Real-Time PCR System.

Testing with the ViraCor 2009 H1N1 Influenza A Real-time RT-PCR test should be performed only on human patients who meet the clinical and epidemiologic criteria for testing suspect specimens. The identification of 2009 H1N1 Influenza A should be performed along with clinical and epidemiological assessment.

Negative results do not preclude influenza virus infection and should not be used as the sole basis for treatment or other patient management decisions.

2. Test Principle

The ViraCor 2009 H1N1 Influenza A Real-time RT-PCR assay is a real-time reverse transcription polymerase chain reaction (rRT-PCR) test. The INFA primer and probe set is designed for detection of matrix RNA from type A Influenza virus in human respiratory samples. The 2009 H1N1 primer and probe set is designed to specifically detect hemagglutinin RNA from 2009 H1N1 Influenza virus in human respiratory samples. The 2009 H1N1 primer and probe set may react with other Influenza A strains of swine origin.

One-step RT-PCR assays are one-tube assays that first reverse-transcribe specific RNA templates into cDNA copies using reverse transcriptase and one of the added oligonucleotide primers to achieve cDNA synthesis. This cDNA then undergoes a polymerase chain reaction (PCR) that utilizes a thermocycling (repeated heating and cooling) of the reaction to logarithmically amplify a specific region of DNA. A dual-labeled fluorogenic hydrolysis (TaqMan) probe anneals to a specific target sequence (and amplicon) located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5′ nuclease activity of Taq polymerase degrades the probe, causing the reporter dye (e.g., FAM) to separate from the quencher dye (e.g., BHQ, Black Hole Quencher), thus generating a fluorescent signal. Additional reporter dye molecules are cleaved from their respective probes with each cycle, proportionately increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle. When the fluorescence intensity exceeds a pre-determined threshold, a cycle threshold (C_T) value is returned for the specific reaction considered.

The purpose of the test is to detect the presence of 2009 H1N1 Influenza A viral RNA in patient respiratory samples. The test should be performed on samples collected from patients exhibiting clinical signs consistent with infection with influenza virus and when a health care professional wishes to establish an etiologic diagnosis. The results for this test should be used in conjunction with clinical findings and should not form the sole basis of a diagnosis or treatment decision.

An Internal Control is included in each sample reaction to ensure that effective nucleic acid extraction has been achieved and to demonstrate the absence of rRT-PCR inhibition.

A positive template control (in vitro transcribed RNA) is included in each rRT-PCR run to ensure that the target Influenza A genes (matrix and hemagglutinin) can be detected by the rRT-PCR assays and is used to demonstrate that the anticipated level of sensitivity has been achieved.

A negative extraction control, which is a known negative sample, is included in each run to ensure that all extraction and amplification reagents are free of target RNA or amplicons and is used to demonstrate that detection of target Influenza genes is not due to false positive results.

3. **Safety Information:** Specimen processing should be performed in accordance with national biological safety regulations.

4. **Materials:**

Reagents:

1. Influenza A (INFA) primer and probe mixture for detection of Influenza A matrix gene and MS2 Internal Control (labeled as "INFA primer/probe mix"). Store rehydrated primers and probes at -20°C or below. Do not store in frost-free freezers.
2. 2009 H1N1 Influenza A (2009 H1N1) primer and probe mixture for detection of 2009 H1N1 Influenza A hemagglutinin gene and MS2 Internal Control (labeled as "2009 H1N1 Influenza A primer/probe mix"). Store rehydrated primers and probes at -20°C or below. Do not store in frost-free freezers.
3. Positive control material – in vitro transcribed RNA: 2 separate positive controls labeled as "INFA" and "2009 H1N1". Store at -20°C or below. Do not store in frost-free freezers.
4. MS2 bacteriophage (5:10,000 dilution), (ATCC catalog # 15597)
5. Total nucleic acid extraction reagents (BioMerieux, catalog # 280140)
    a. NucliSENS Lysis Buffer
    b. NucliSENS Extraction Buffer 1
    c. NucliSENS Extraction Buffer 2
    d. NucliSENS Extraction Buffer 3
    e. NucliSENS Magnetic Silica

5. Superscript III Platinum one-step RT-PCR reagents (Invitrogen, Inc., catalog #11732-088)
    a. SuperScript™ III RT/Platinum® Taq Mix
    b. 2X Reaction Mix (a buffer containing 0.4 mM of each dNTP and 6 mM $MgSO_4$)
    c. 50 mM Magnesium Sulfate ($MgSO_4$)
    d. ROX Reference Dye (25 µM)
6. Sterile Nuclease-free water

Supplies:

1. NucliSENS easyMAG disposables
2. RNase/DNase-free 5 mL tubes
3. RNase/DNase-free 1.5 mL polypropylene microcentrifuge tubes and racks
4. Sterile, RNase/DNase-free disposable aerosol-barrier micropipettor tips
5. RNase/DNase-free 96 well thermocycler plate rack or appropriate tube rack
6. 96-well optical reaction plate or other reaction vessels
7. Optical adhesive cover (for use with 96-well optical reaction plate)
8. Appropriate personal protective equipment (PPE) including disposable, powder-free gloves

Equipment:

1. Class II Biosafety cabinet (BSC) for specimen handling prior to extraction
2. Real-Time PCR System: Applied Biosystems 7500 Real-Time standard mode PCR System, SDS software version 1.4.
3. NucliSENS® easyMag™ extraction platform, software version 2.0
4. Single or multi-channel micropipette(s) with an accuracy range between 1-10 µL, 10-100 µL and 100-1000 µL. NOTE: A separate set of micropipettes is required for extraction, sample addition and amplification areas.
5. epMotion 5070 or 5075 automated pipetting system if using automated plating option
6. Freezer (manual defrost) at -10 to -30°C (for kit component frozen storage)
7. Freezer (manual defrost) at -10 to -30°C (for specimen frozen storage)
8. Refrigerator at 2 to 8°C.
9. Bench top centrifuge for low speed centrifugation of 96-well plates or other reaction vessels
10. Microcentrifuge
11. Vortex mixer
12. Cooler racks for 1.5 mL microcentrifuge tubes and 96-well 0.2 mL PCR reaction tubes or plates.

The test is assembled at ViraCor Laboratories and performed by ViraCor personnel consistent with practices for the preparation of PCR assay reagent systems based on Clinical Laboratory Improvement Amendments (CLIA) standards.

5. Procedure:

Contamination prevention:

1. Maintain separate areas for specimen extraction, assay setup, and handling of nucleic acids.
2. Maintain separate, dedicated equipment and supplies for assay setup and handling of extracted nucleic acids.
3. Wear a separate lab coat for specimen extraction and nucleic acid handling and assay setup.
4. Change gloves between samples and whenever contamination is suspected.
5. Keep reagent and reaction tubes capped or covered when possible.
6. Clean work surfaces daily with RNase Away® or 5% bleach to minimize risk of nucleic acid contamination.

Nucleic Acid Extraction: Use a dedicated area for specimen extraction

1. Nucleic acids are extracted from specimens using the bioMerieux NucliSENS easyMAG platform. Refer to the manufacturer's User Manual for total nucleic acid extraction using this platform. Either the 'on-board' or 'off-board' workflows may be utilized for sample extraction.
2. 400 µl should be selected for the sample volume (360 µl of specimen and 20 µl of MS2 internal control) and the elution should be set for 100 µl.

Reagent preparation: (Note: Keep all reagents in a cold block during assay set-up.)

1. Primers and probes
    a. Thaw frozen aliquots of primers and probes. (Thawed aliquots of probes may be stored in the dark for up to 2 months at 4°C. Do not re-freeze oligonucleotides).
    b. Vortex all primer and probe mixes.
    c. Place primer and probe mixes into cold block.
2. Real-Time RT-PCR reagents
    a. Thaw 2X buffer, ROX passive reference dye (diluted 1:10), molecular grade water.
    b. Vortex 2X buffer and ROX passive reference dye (diluted 1:10) and place in cold block.
    c. Place water and enzyme into cold block.

Tests for each RT-PCR run:

1. Each sample RNA extract is tested by separate primer/probe sets: INFA with MS2 and 2009 H1N1 with MS2. The MS2 primer and probe set targets the MS2 bacteriophage added to each specimen prior to extraction and thus serves as an internal positive control for both sample extraction and amplification.
2. A negative extraction control (NEC) is included on each extraction run and is analyzed for each primer/probe set on each RT-PCR run. A positive template control (POS) is included for each primer/probe set on each RT-PCR run.

Reaction Setup: Assay reaction mixtures are made as a cocktail and dispensed into the 96-well reaction plate. Extracted nucleic acid or positive template controls are then added to the appropriate test reactions and controls.

1. Label one 5 mL tube for each primer/probe set.
2. Determine the number of reactions to set up per assay. It is necessary to make excess reaction mixture to allow for the NEC and POS reactions and pipetting error.
3. Master Mix: calculate the amount of each reagent to be added for each primer/probe set reaction master mix. The calculations are as follows:

Table 1: Volumes of reagents in reaction mixtures

	INFA / MS2 Mix				**2009 H1N1 / MS2 Mix**			
Reagent	µl / reaction	x	wells	= total	µl / reaction	x	wells	= total
2X Buffer	25	x			25	x		
Primer/Probe	5	x			5	x		
Enzyme	1	x			1	x		
ROX 1:10	1	x			1	x		
Water	8	x			8	x		
Total	40	x			40	x		

4. After the addition of the water, vortex the reaction mixtures and centrifuge briefly and place tube in a cold rack.
5. Set up reaction plate in a 96-well cooler rack and dispense 40 µl of each master mix into each well going across the row as shown below. Alternatively, epMotion automated pipetting system may be used to dispense mixes.
6. Transfer the reaction plate to the nucleic acid handling area.
7. Briefly vortex and centrifuge the tubes containing the samples.
8. Add samples to the reaction plate as shown below. Pipette 10 µl of the first sample into each well labeled for that sample changing tips after each addition. Alternatively, epMotion automated pipetting system may be used to dispense samples.

Table 2: Example Test Set-up

	1	2	3	4	5	6	7	8	9	10	11	12
A	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA
B	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA
C	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA
D	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA
E	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA
F	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA
G	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA
H	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA	2009 H1N1	INFA

Table 3: Example Sample Set-up

A	NEC 1	NEC1	Sample 8	Sample 8	Sample 16	Sample 16	NEC 2	NEC 2	Sample 31	Sample 31	Sample 39	Sample 39
B	Sample 1	Sample 1	Sample 9	Sample 9	Sample 17	Sample 17	Sample 24	Sample 24	Sample 32	Sample 32	Sample 40	Sample 40
C	Sample 2	Sample 2	Sample 10	Sample 10	Sample 18	Sample 18	Sample 25	Sample 25	Sample 33	Sample 33	Sample 41	Sample 41
D	Sample 3	Sample 3	Sample 11	Sample 11	Sample 19	Sample 19	Sample 26	Sample 26	Sample 34	Sample 34	Sample 42	Sample 42
E	Sample 4	Sample 4	Sample 12	Sample 12	Sample 20	Sample 20	Sample 27	Sample 27	Sample 35	Sample 35	Sample 43	Sample 43
F	Sample 5	Sample 5	Sample 13	Sample 13	Sample 21	Sample 21	Sample 28	Sample 28	Sample 36	Sample 36	Sample 44	Sample 44
G	Sample 6	Sample 6	Sample 14	Sample 14	Sample 22	Sample 22	Sample 29	Sample 29	Sample 37	Sample 37	Sample 45	Sample 45
H	Sample7	Sample7	Sample 15	Sample 15	Sample 23	Sample 23	Sample 30	Sample 30	Sample 38	Sample 38	H1N1 POS	INFA POS
High and low level controls are run on alternate days												

9. Cover the reaction plate with an adhesive seal.
10. Briefly vortex the reaction plate.
11. Centrifuge the reaction plate for 30 seconds to bring the reaction mixture to the bottom of the well.
12. Return plate to cold block.

RT-PCR Amplification Conditions:

1. Turn on the ABI laptop, ABI instrument and open the ABI software.
2. In the Set Up>Plate window, use the well inspector (View -> Well Inspector) to modify settings for each well to include Sample ID and appropriate detectors for each analyte as shown below.

Table 4: Summary information for INFA and 2009 H1N1 analytes

INFA	INFA	MS2
Reporter - Quencher	FAM-none	VIC-TAMRA

2009 H1N1	2009 H1N1	MS2
Reporter - Quencher	FAM-none	VIC-TAMRA

3. Verify that the ABI is set up in “standard mode” using the following thermocycling parameters.

Table 5: Summary of thermocycling parameters

Stage	Cycle Reps Number	Temperature	Duration
1 Reverse Transcription	1	50°C	15 minutes
2 Taq Inhibitor Activation	1	95°C	2 minutes
3 PCR amplification	45	95°C	15 seconds
		60°C	33 seconds

4. Place plate in instrument.
5. Select start and remove gloves.
6. When run is complete, press OK.
7. From Results>Amplification Plot Tab, set the threshold at 0.1 and the baseline to manual.

Interpretation / Examination:

1. Verify that the NEC signal(s) are below the threshold for the FAM-none targets (FAM channel). If a false positive occurs, sample contamination may have occurred. Repeat from extraction step all specimens included in the extraction run with the NEC with stricter adherence to the procedure guidelines.
2. Verify that the POS control C_T values (FAM channel) are within the established reference ranges for the INFA and 2009 H1N1 targets listed below:

Table 6: C_T Ranges for High and Low Positive Controls

	High Positive (C_T)	Low Positive (C_T)
INFA	22.0-26.0	29.0-33.0
2009 H1N1	22.5-26.5	29.5-33.5

If the INFA or 2009 H1N1 Positive Control reaction curves do not cross the threshold line within the expected ranges, invalidate the run and repeat the assay (extraction and RT-PCR) with stricter adherence to procedure guidelines.

3. All clinical samples should exhibit MS2 reaction curves (VIC channel) that cross the threshold line at or before cycle 35 when the INFA or 2009 H1N1 reaction curves cross the threshold at or after cycle 40. When both the INFA and 2009 H1N1 reaction curves cross the threshold before cycle 40, competition for reagents may occur with the MS2 reaction and thus the C_T of this reaction is not evaluated in samples that are positive for both INFA and 2009 H1N1 reactions.

4. When all controls meet stated requirements, a specimen is scored ***Detected*** for Influenza A viral RNA if the INFA reaction curve crosses the threshold line within 40 cycles (FAM channel). If the reaction for Influenza A is positive, it may also be positive for 2009 H1N1 (FAM channel). A patient specimen is scored ***Detected*** for 2009 H1N1 Influenza A viral RNA if both the INFA and 2009 H1N1 reaction curves cross the threshold at or before cycle 40. A patient specimen is scored ***Not Detected*** for Influenza viral RNA if curves for neither the INFA nor the 2009 H1N1 reactions cross the threshold within 40 cycles. See Table 7 below:

Table 7: Summary of Interpretation of Results

INFA C_T	**2009 H1N1 C_T**	**IC C_T**	**Reported Result**
≤ 40	≤ 40	N/A	Influenza A RNA: Detected 2009 H1N1 RNA: Detected
> 40 or no amplification	> 40 or no amplification	≤ 35	Influenza A RNA: Not Detected 2009 H1N1 RNA: Not Detected
≤ 40	> 40 or no amplification	≤ 35	Influenza A RNA: Detected 2009 H1N1 RNA: Not Detected
> 40 or no amplification	≤ 40	≤ 35	Repeat testing required; If same results on repeat: Indeterminate, see comment 2
≤ 40	> 40 or no amplification	>35	Repeat testing required; If same results on repeat: Indeterminate, see comment 1
> 40 or no amplification	≤ 40	>35	Repeat testing required; If same results on repeat: Indeterminate, see comment 1
> 40 or no amplification	> 40 or no amplification	>35	Repeat testing required; If same results on repeat: Indeterminate, see comment 1

Comment 1: Presence of assay inhibition detected, which precluded interpretation of results.
Comment 2: Discordant results observed (2009 H1N1 viral RNA was detected; however Influenza A viral RNA was not detected)

6. Limitations

1. Individuals performing analyses should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
2. Results from this test must be interpreted in conjunction with the clinical history, epidemiological data, clinical signs and symptoms, and other data available to the clinician evaluating the patient.
3. The prevalence of infection will affect the test's predictive value.
4. Performance of this test for detection of strains of swine influenza virus other than 2009 H1N1 has not been established.

5. As with other tests, negative results do not rule out Influenza A or 2009 H1N1 influenza infection, and should not be used as the sole basis for patient clinical management decisions.
6. A false negative result may occur due to assay inhibitors or other types of interference.
7. A false negative result may occur when the infecting organism has genomic mutations, insertions, deletions, or rearrangements; if the virus mutates in the target region, 2009 H1N1 influenza virus may not be detected or may be detected less predictably.
8. A false negative result may occur when performed very early in the course of illness.
9. A false negative result may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling.
10. There is a risk of false positive values resulting from specimen cross-contamination by target organisms or their nucleic acid.
11. The performance of this test has not been established for monitoring treatment of 2009 H1N1 influenza infection.
12. The performance of this test has not been established for screening of blood or blood product for the presence of influenza A or 2009 H1N1 influenza.
13. This test is a qualitative test and does not provide the quantitative value of detected organism present.
14. This test cannot rule out diseases caused by other bacterial or viral pathogens.
15. The use of sputum has not been evaluated with this assay.

7. Assay Performance

Analytical Sensitivity

Two studies were carried out:

In Study 1, the Limit of Detection (LOD) was determined for INFA and 2009 H1N1 detectors using 2009 H1N1, seasonal H1N1 (A1/MaI/302/54), and seasonal H3N2 (A/Victoria/3/75) viruses diluted in an upper respiratory sample matrix (nasal wash) and in a lower respiratory sample matrix (BAL). The 2009 H1N1 influenza virus was obtained from a clinical sample and identified as Influenza A unsubtypable by xTAG® RVP (Luminex Corporation) and confirmed as 2009 H1N1 influenza virus by the Focus Diagnostics Influenza A H1N1 (2009) Real Time RT-PCR assay. An initial estimate of LOD was obtained using serial dilutions of viruses tested in four replicates for each dilution. The limit of detection was confirmed by extraction and amplification of 20 replicates. The LOD was determined to be the dilution at which at least 95% of the replicates were positive. The LOD data for Study 1 are summarized in Tables 8A, 9A, 10A, 11A, 12A and 13A below.

In Study 2, the ViraCor 2009 H1N1 Influenza A Real-time RT-PCR assay was compared with an alternative test (comparator test) previously authorized by the FDA for detection of 2009 H1N1 influenza virus. Serial dilutions of the same viruses used in Study 1 were made in upper and lower respiratory matrices and each dilution tested in five replicates. Samples for use with the comparator assay were extracted using the appropriate extraction method specified for this assay. Results from Study 2 are summarized in Tables 8B, 9B, 10B, 11B, 12B and 13B below.

Table 8A: Summary of LOD results for 2009 H1N1 influenza virus in nasal wash matrix.

Est. gene copies/mL	ViraCor assay			
	INFA		2009 H1N1	
	Initial	Confirm	Initial	Confirm
10,000	4/4	N/A	4/4	N/A
1,000	4/4	20/20	4/4	20/20
700	4/4	N/A	4/4	N/A
500	4/4	20/20	1/4	19/20
200	4/4	N/A	4/4	N/A
100	2/4	N/A	3/4	N/A

LOD of ViraCor assay for 2009 H1N1 virus in nasal wash was determined to be 500 copies per mL.

Table 8B: Comparison of ViraCor assay to Comparator test using 2009 H1N1 influenza virus in nasal wash matrix.

	ViraCor Assay		Comparator			
Est. gene copies/mL	INFA	2009 H1N1	Flu A	H1N1-1	H1N1-2	QIPC
1,000	5/5	5/5	5/5	5/5	5/5	5/5
500	5/5	5/5	5/5	4/5	5/5	5/5
200	5/5	5/5	4/5	5/5	4/5	5/5
100	5/5	5/5	3/5	3/5	1/5	5/5

The ViraCor assay is at least equivalent in sensitivity to the Comparator test for 2009 H1N1 influenza virus in nasal wash.

Table 9A: Summary of LOD results for 2009 H1N1 influenza virus in BAL matrix.

Est. gene copies/mL	ViraCor assay			
	INFA		2009 H1N1	
	Initial	Confirm	Initial	Confirm
10,000	4/4	N/A	4/4	N/A
1,000	4/4	20/20	4/4	20/20
700	4/4	N/A	4/4	N/A
500	4/4	20/20	1/4	20/20
200	4/4	N/A	4/4	N/A
100	2/4	N/A	3/4	N/A

LOD of ViraCor assay for 2009 H1N1 virus in BAL was determined to be 500 copies per mL.

Table 9B: Comparison of ViraCor assay to Comparator test using 2009 H1N1 influenza virus in BAL.

	ViraCor Assay		Comparator			
Est. gene copies/mL	INFA	2009 H1N1	Flu A	H1N1-1	H1N1-2	QIPC
1,000	5/5	5/5	5/5	5/5	5/5	5/5
500	5/5	5/5	5/5	5/5	1/5	5/5
200	5/5	5/5	5/5	5/5	0/5	5/5
100	5/5	5/5	5/5	5/5	0/5	5/5

The ViraCor assay is at least equivalent in sensitivity to the Comparator test for 2009 H1N1 influenza virus in BAL.

Table 10A: Summary of LOD results for seasonal H1N1 Influenza A virus in nasal wash.

Est. gene copies/mL	$TCID_{50}$/mL	ViraCor assay			
		INFA		2009 H1N1	
		Initial	Confirm	Initial	Confirm
10,000	2,189	4/4	N/A	0/4	N/A
1,000	219	4/4	20/20	0/4	0/20
700	153	4/4	N/A	0/4	N/A
500	109	4/4	20/20	0/4	0/20
200	44	1/4	N/A	0/4	N/A
100	22	2/4	N/A	0/4	N/A

LOD of ViraCor assay for seasonal H1N1 virus in nasal wash was determined to be 500 copies per mL or 109 $TCID_{50}$/mL.

Table 10B: Comparison of ViraCor assay to Comparator test using seasonal H1N1 influenza A virus in nasal wash.

	ViraCor Assay		Comparator			
Est. gene copies/mL	INFA	2009 H1N1	Flu A	H1N1-1	H1N1-2	QIPC
1,000	5/5	0/5	1/5	0/5	0/5	5/5
500	5/5	0/5	0/5	0/5	0/5	5/5
200	5/5	0/5	0/5	0/5	0/5	5/5
100	5/5	0/5	0/5	0/5	0/5	5/5

The ViraCor assay is at least equivalent in sensitivity to the Comparator test for seasonal H1N1 influenza A virus in nasal wash.

Table 11A: Summary of LOD results for seasonal H1N1 Influenza A virus in BAL.

Est. gene copies/mL	$TCID_{50}$/mL	ViraCor assay			
		INFA		2009 H1N1	
		Initial	Confirm	Initial	Confirm
10,000	2,189	4/4	N/A	0/4	N/A
1,000	219	4/4	20/20	0/4	0/20
700	153	4/4	N/A	0/4	N/A
500	109	4/4	19/20	0/4	0/20
200	44	1/4	N/A	0/4	N/A
100	22	2/4	N/A	0/4	N/A

LOD of ViraCor assay for seasonal H1N1 virus in BAL was determined to be 500 copies per mL or 109 $TCID_{50}$/mL.

Table 11B: Comparison of ViraCor assay to Comparator test using seasonal H1N1 influenza A virus in BAL.

	ViraCor Assay		Comparator			
Est. gene copies/mL	INFA	2009 H1N1	Flu A	H1N1-1	H1N1-2	QIPC
1,000	5/5	0/5	5/5	0/5	0/5	5/5
500	5/5	0/5	5/5	0/5	0/5	5/5
200	5/5	0/5	4/5	0/5	0/5	5/5
100	5/5	0/5	4/5	0/5	0/5	5/5

The ViraCor assay is at least equivalent in sensitivity to the Comparator test for seasonal H1N1 influenza A virus in BAL.

Table 12A: Summary of LOD results for seasonal H3N2 Influenza A virus in nasal wash.

Est. gene copies/mL	$TCID_{50}$/mL	ViraCor assay			
		INFA		2009 H1N1	
		Initial	Confirm	Initial	Confirm
10,000	34	4/4	N/A	0/4	N/A
1,000	3	4/4	20/20	0/4	0/20
700	2	4/4	N/A	0/4	N/A
500	2	4/4	20/20	0/4	0/20
200	0.7	3/4	N/A	0/4	N/A
100	0.3	0/4	N/A	0/4	N/A

LOD of ViraCor assay for seasonal H3N2 virus in nasal wash was determined to be 500 copies per mL or 2 $TCID_{50}$/mL.

Table 12B: Comparison of ViraCor assay to Comparator test using seasonal H3N2 influenza A virus in nasal wash.

	ViraCor Assay		Comparator			
Est. gene copies/mL	INFA	2009 H1N1	Flu A	H1N1-1	H1N1-2	QIPC
1,000	5/5	0/5	2/5	0/5	0/5	5/5
500	5/5	0/5	4/5	0/5	0/5	5/5
200	5/5	0/5	0/5	0/5	0/5	5/5
100	4/5	0/5	1/5	0/5	0/5	5/5

The ViraCor assay is at least equivalent in sensitivity to the Comparator test for seasonal H3N2 influenza A virus in nasal wash.

Table 13A: Summary of LOD results for seasonal H3N2 Influenza A virus in BAL.

Est. gene copies/mL	$TCID_{50}$/mL	ViraCor assay			
		INFA		2009 H1N1	
		Initial	Confirm	Initial	Confirm
10,000	34	4/4	N/A	0/4	N/A
1,000	3	4/4	20/20	0/4	0/20
700	2	4/4	N/A	0/4	N/A
500	2	4/4	20/20	0/4	0/20
200	0.7	3/4	N/A	0/4	N/A
100	0.3	0/4	N/A	0/4	N/A

LOD of ViraCor assay for seasonal H3N2 virus in BAL was determined to be 500 copies per mL or 2 $TCID_{50}$/mL.

Table 13B: Comparison of ViraCor assay to Comparator test using seasonal H3N2 influenza A virus in BAL.

	ViraCor Assay		Comparator			
Est. gene copies/mL	INFA	2009 H1N1	Flu A	H1N1-1	H1N1-2	QIPC
1,000	5/5	0/5	5/5	0/5	0/5	5/5
500	5/5	0/5	5/5	0/5	0/5	5/5
200	5/5	0/5	4/5	0/5	0/5	5/5
100	5/5	0/5	2/5	0/5	0/5	5/5

The ViraCor assay is at least equivalent in sensitivity to the Comparator test for seasonal H3N2 influenza A virus in BAL.

Repeatability

Intra-assay and inter-assay precision studies demonstrated the reproducibility (repeatability) of the ViraCor 2009 H1N1 Influenza A Real-time RT-PCR assay. Three replicates at three dilutions were tested by both INFA and 2009 H1N1 reactions on three different days. The mean, standard deviation (Stnd Dev), 95% confidence interval (95%CI) and coefficient of variation (expressed as a percentage, %CV) of the C_T values were determined for each of the day 1, day 2 and day 3 runs and represent the intra-assay precision. The same parameters were determined for all C_T values of each dilution and represent the inter-assay precision of the assays. Intra-assay and inter-assay precision is shown in Table 14.

Table 14: Intra- and inter-assay precision of the ViraCor INFA and 2009 H1N1 reactions in nasal wash and BAL matrices

		Nasal Wash (NW)						Bronchoalveolar Lavage (BAL)					
		Inf A matrix C_T value			2009 H1N1 C_T value			Inf A matrix C_T value			2009 H1N1 C_T value		
Run	**Replic. #**	**Dil. 1**	**Dil. 2**	**Dil. 3**	**Dil. 1**	**Dil. 2**	**Dil. 3**	**Dil. 1**	**Dil. 2**	**Dil. 3**	**Dil. 1**	**Dil. 2**	**Dil. 3**
Intra-assay precision (day 1)	1	26.09	29.77	34.91	26.69	30.60	33.70	27.33	31.91	33.73	29.30	34.50	37.14
	2	25.97	30.12	32.89	26.30	31.08	34.07	27.05	31.96	35.12	29.03	34.51	38.54
	3	25.81	28.96	32.08	26.43	30.07	34.70	27.60	31.76	34.78	29.45	34.13	36.05
	Mean	25.96	29.61	33.29	26.47	30.58	34.16	27.33	31.88	34.55	29.26	34.38	37.24
	Stnd Dev	0.14	0.60	1.46	0.20	0.51	0.50	0.28	0.10	0.73	0.21	0.22	1.25
	95%CI	0.16	0.68	1.65	0.23	0.58	0.57	0.31	0.12	0.82	0.24	0.25	1.42
	%CV	0.54%	2.02%	4.38%	0.75%	1.67%	1.47%	1.01%	0.33%	2.11%	0.73%	0.63%	3.36%
Intra-assay precision (day 2)	1	26.15	30.14	34.05	27.57	31.29	35.04	25.45	29.42	32.49	27.46	31.40	34.54
	2	25.94	30.13	34.06	27.50	31.43	34.21	25.46	29.09	33.05	26.73	30.44	34.22
	3	25.55	29.49	33.52	27.35	31.38	35.07	25.72	29.20	34.07	27.34	29.61	34.47
	Mean	25.88	29.92	33.87	27.47	31.36	34.77	25.54	29.23	33.21	27.17	30.49	34.41
	Stnd Dev	0.30	0.37	0.31	0.11	0.07	0.49	0.15	0.17	0.80	0.39	0.90	0.17
	95%CI	0.34	0.42	0.35	0.13	0.08	0.55	0.17	0.19	0.91	0.44	1.02	0.19
	%CV	1.16%	1.25%	0.91%	0.41%	0.23%	1.40%	0.60%	0.57%	2.41%	1.45%	2.95%	0.48%
Intra-assay precision (day 3)	1	25.37	29.07	32.93	25.22	29.32	31.72	27.05	29.66	32.45	27.99	30.71	33.49
	2	25.27	29.05	32.43	25.43	29.38	31.28	26.66	30.52	31.69	28.17	31.17	34.78
	3	24.83	28.66	31.65	25.80	30.32	34.67	26.77	30.31	33.76	28.53	30.77	35.15
	Mean	25.15	28.93	32.34	25.48	29.67	32.56	26.83	30.16	32.64	28.23	30.89	34.47
	Stnd Dev	0.29	0.23	0.65	0.29	0.56	1.84	0.20	0.45	1.05	0.28	0.25	0.87
	95%CI	0.33	0.26	0.73	0.33	0.63	2.09	0.22	0.51	1.19	0.31	0.28	0.99
	%CV	1.15%	0.81%	2.00%	1.15%	1.88%	5.66%	0.74%	1.49%	3.21%	0.98%	0.80%	2.53%
Inter-assay precision	**Mean**	**25.66**	**29.49**	**33.17**	**26.48**	**30.54**	**33.83**	**26.56**	**30.42**	**33.46**	**28.22**	**31.92**	**35.37**
	Stnd Dev	**0.44**	**0.57**	**1.06**	**0.88**	**0.82**	**1.40**	**0.82**	**1.19**	**1.13**	**0.94**	**1.92**	**1.60**
	95%CI	**0.29**	**0.38**	**0.69**	**0.58**	**0.54**	**0.91**	**0.54**	**0.78**	**0.74**	**0.61**	**1.25**	**1.04**
	%CV	**1.72%**	**1.95%**	**3.18%**	**3.33%**	**2.70%**	**4.13%**	**3.09%**	**3.90%**	**3.39%**	**3.33%**	**6.00%**	**4.51%**

Analytical Specificity

Inclusivity

Nine different strains of Influenza A at levels close to LOD were tested with the ViraCor 2009 H1N1 Influenza A Real-time RT-PCR assay. The INFA detector gave positive results for all nine strains. The 2009 H1N1 detector gave negative results for eight of the nine strains and gave a positive result for Influenza A/New Jersey/8/76 which is a strain of swine origin. The results are summarized in Table 15.

Table 15: Analytical Inclusivity Results for ViraCor 2009 H1N1 Influenza A Real-time RT-PCR assay[a]

Viral Strain	Subtype	Concentration ($TCID_{50}$/mL)	INFA	2009 H1N1
A/NWS/33	H1N1	1.6	+	-
A1/Denver/1/57	H1N1	88.9	+	-
A/New Jersey/8/76 (Hsw N1)	H1N1	150	+	+[b]
A/PR/8/34	H1N1	88.9	+	-
A/Mal/302/54	H1N1	890	+	-
A/Hong Kong/8/68	H3N2	88.9	+	-
A2/Aichi/2/68	H3N2	15.8	+	-
A/Port Chalmers/1/73	H3N2	15.8	+	-
A/Victoria/3/75	H3N2	0.9	+	-

[a] The same results were obtained when testing was carried out with the same strains of virus tested at levels 100-fold greater than those listed in Table 15.
[b] The ViraCor 2009 H1N1 Influenza A Real-time RT-PCR assay is reactive with Influenza A/New Jersey/8/76 which is a strain of swine origin isolated in 1976.

Cross-reactivity

Cross-reactivity of the ViraCor 2009 H1N1 Influenza A Real-time RT-PCR assay was evaluated using other human respiratory and non-respiratory pathogens. Genomic RNA or DNA of different organisms was extracted using NucliSENS® easyMag™ total nucleic acid extraction reagents and assayed to show no cross-reactivity of each primer and probe set with nucleic acids of other organisms. Results are shown in Tables 16A and 16B.

Table 16A: Assessment of assay specificity with non-Influenza A human respiratory and non-respiratory pathogens

DNA Template Source	Source of pathogen	C_T[a] (INFA)	C_T (2009 H1N1)	Copies/mL
Adenovirus	ATCC	N.D.[b]	N.D.	1.7×10^6
BK virus	ATCC	N.D.	N.D.	5.5×10^5
Cytomegalovirus	ATCC	N.D.	N.D.	6.7×10^5
Epstein-Barr virus	ATCC	N.D.	N.D.	2.7×10^5
Human herpesvirus 6	ATCC	N.D.	N.D.	8.5×10^5
Human herpesvirus 7	Clinical Sample	N.D.	N.D.	4.9×10^5
Human herpesvirus 8	Clinical Sample	N.D.	N.D.	4.2×10^6
Herpes simplex virus	ATCC	N.D.	N.D.	7.2×10^5
JC virus	ATCC	N.D.	N.D.	4.9×10^7
Parvovirus B19	Clinical Sample	N.D.	N.D.	1.2×10^9
Pneumocystis jiroveci	ATCC	N.D.	N.D.	3.9×10^5
SV40 virus	ATCC	N.D.	N.D.	2.7×10^6
Toxoplasma gondii	ATCC	N.D.	N.D.	5.3×10^5
Varicella-Zoster virus	ATCC	N.D.	N.D.	1.2×10^6
Hepatitis B virus	ATCC	N.D.	N.D.	3.7×10^6
Hepatitis C virus	Acrometrix	N.D.	N.D.	1.1×10^6
Human enterovirus	ATCC	N.D.	N.D.	1.0×10^6
Chlamydophila pneumoniae	ATCC	N.D.	N.D.	1.5×10^6
Mycoplasma pneumoniae	ATCC	N.D.	N.D.	7.3×10^4
Legionella pneumophila	ATCC	N.D.	N.D.	4.0×10^6
No template controls	N/A[c]	N.D.	N.D.	N/A

[a]C_T, cycle threshold, [b]N.D., Not detected, [c]N/A, Not applicable

Table 16b: Assessment of assay specificity with non-Influenza A human respiratory and non-respiratory pathogens analyzed by xTAG-RVP

DNA Template Source	Source of pathogen	C_T[a] (INFA)	C_T (2009 H1N1)	xTAG-RVP result
ParaInfluenza 1	Zeptometrix	N.D.[b]	N.D.	Positive, Para1
ParaInfluenza 2	Zeptometrix	N.D.	N.D.	Positive, Para 2
ParaInfluenza 3	Zeptometrix	N.D.	N.D.	Positive, Para3
Rhinovirus	Zeptometrix	N.D.	N.D.	Positive, Rhinovirus
Enterovirus	Zeptometrix	N.D.	N.D.	Positive, Enterovirus/Rhinovirus
Adenovirus	Zeptometrix	N.D.	N.D.	Positive, Adenovirus
Respiratory syncytial virus A	Zeptometrix	N.D.	N.D.	Positive, RSVA
Respiratory syncytial virus B	Zeptometrix	N.D.	N.D.	Positive, RSVB
Human Metapneumovirus	Zeptometrix	N.D.	N.D.	Positive, hMPV
Influenza B	Clinical Sample[c]	N.D.	N.D.	Positive, Influenza B
Influenza B	Clinical Sample	N.D.	N.D.	Positive, Influenza B
Influenza B	Clinical Sample	N.D.	N.D.	Positive, Influenza B
Influenza B	Clinical Sample	N.D.	N.D.	Positive, Influenza B
Respiratory syncytial virus A	Clinical Sample	N.D.	N.D.	Positive, RSVA
Respiratory syncytial virus A	Clinical Sample	N.D.	N.D.	Positive, RSVA
Respiratory syncytial virus A	Clinical Sample	N.D.	N.D.	Positive, RSVA
Respiratory syncytial virus A	Clinical Sample	N.D.	N.D.	Positive, RSVA
Respiratory syncytial virus A	Clinical Sample	N.D.	N.D.	Positive, RSVA
Respiratory syncytial virus B	Clinical Sample	N.D.	N.D.	Positive, RSVB
Respiratory syncytial virus A, Respiratory syncytial virus B	Clinical Sample	N.D.	N.D.	Positive, RSVA, RSVB
Respiratory syncytial virus B	Clinical Sample	N.D.	N.D.	Positive, RSVB
Respiratory syncytial virus B	Clinical Sample	N.D.	N.D.	Positive, RSVB
Respiratory syncytial virus B	Clinical Sample	N.D.	N.D.	Positive, RSVB
Coronavirus VR-70	ATCC	N.D.	N.D.	Positive, Coronavirus 229E
ParaInfluenza 4	Clinical Sample	N.D.	N.D.	Positive, ParaInfluenza 4
No template controls	N/A[d]	N.D.	N.D.	N/A

[a]C_T, cycle threshold, [b]N.D., Not detected, [c]ViraCor Laboratories positive clinical specimen,
[d]N/A, Not applicable

Clinical Studies

Clinical performance of the ViraCor 2009 H1N1 Influenza A Real-time RT-PCR assay was assessed against a comparator assay which had been previously authorized by the FDA for detection of 2009 H1N1 influenza virus under an Emergency Use Authorization. For this analysis, the samples used were residual specimens obtained from ViraCor's clinical caseload initially submitted for analysis by the FDA-cleared xTAG-RVP assay. Presumptive positive samples were those with the result of "Influenza A positive, unsubtypable" by the xTAG-RVP assay. Presumptive negative samples were those with a result of "not detected" for both Influenza A and seasonal H1 or seasonal H3 subtypes.

A summary of the comparative results between the ViraCor 2009 H1N1 Influenza A Real-time RT-PCR assay and the authorized comparator is shown in Table 17 and 18.

Table 17: Summary of clinical performance evaluation: nasal swab and wash samples

		FDA Authorized Comparator Assay			
		2009 H1N1 positive	**2009 H1N1 negative**	**Total**	
ViraCor Assay	**2009 H1N1 positive**	20	0	20	**100% (20/20) positive agreement (95%CI 83.2% - 100%)**
	2009 H1N1 negative	0	50	50	**100% (50/50) negative agreement (95%CI 92.9% - 100%)**
	Total	20	50		

Table 18: Summary of clinical performance evaluation: lower respiratory samples (BAL)

		FDA Authorized Comparator Assay			
		2009 H1N1 positive	**2009 H1N1 negative**	**Total**	
ViraCor Assay	**2009 H1N1 positive**	10	0	10	**100% (10/10) positive agreement (95%CI % 69.2% - 100%)**
	2009 H1N1 negative	0	50	50	**100% (50/50) negative agreement (95%CI 92.9% - 100%)**
	Total	10	50		

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